

In vitro regeneration of *Senna alata* (L.) Robx. (Fabaceae): An ancient medicinal plant

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ABSTRACT: *Senna alata*, a legume of medicinal importance, presents antifungal and antibacterial antioxidant activity. The main limitation is the presence of seeds with integument dormancy within natural populations. This study aimed to establish an efficient protocol for the *in vitro* propagation and acclimatization of seedlings. The seeds were disinfected with different concentrations of NaOCl; for the regeneration of the shoots, cotyledon segments were inoculated in MS and WPM medium, supplemented with plant growth regulators, auxins (1-NAA and 2,4-D), interaction with cytokinins (TDZ and BAP), and activated carbon. The seedlings were acclimated in nursery conditions using vermiculite, sand, and soil substrates. Using NaOCl (0.5%) for 30 min resulted in 98.0% disinfected seeds while 95.4% of scarified seeds germinated. High node and shoots numbers (8.3 ± 0.4 ; 12.6 ± 0.8 , respectively) were obtained when using the MS medium containing 1-NAA + TDZ ($0.1 + 1.0 \text{ mg L}^{-1}$), while the use of MS medium supplemented with 2,4-D + TDZ ($0.1 + 1.0 \text{ mg L}^{-1}$) resulted in longer shoots ($6.0 \pm 0.5 \text{ cm}$). Using WPM medium with IBA + BAP ($1.0 + 0.1 \text{ mg L}^{-1}$) improved rooting ($14.0 \text{ cm} \pm 1.3$) and the number of roots (5.0 ± 0.0) in the treated plants. The vermiculite substrate with sand promoted 81.5% of acclimatized plants. This study allowed obtaining high levels of germination, regeneration *in vitro* and acclimatization of *S. alata*.

Regeneração *in vitro* de *Senna alata* (L.) Robx. (Fabaceae), uma antiga planta medicinal

RESUMO: *Senna alata*, leguminosa de importância medicinal, apresenta atividade antioxidante antifúngica e antibacteriana, tendo como principal limitação a presença de sementes com dormência tegumentar em populações naturais. O estudo teve como objetivo estabelecer um protocolo *in vitro* eficiente para a propagação *in vitro* e aclimação de mudas. As sementes foram desinfetadas com diferentes concentrações de NaOCl, para a regeneração dos brotos, segmentos cotiledonares foram inoculados em meio MS e WPM, suplementados com reguladores de crescimento de plantas, auxinas (1-NAA e 2,4-D), interação com citocininas (TDZ e BAP) e carvão ativado. As mudas foram aclimatadas em viveiro, utilizando como substrato de vermiculita, areia e solo. O uso de NaOCl (0,5%) / 30 min resultou em 98,0 % de sementes desinfetadas. As sementes escarificadas resultaram em 95,4% de germinação. Maiores valores de indução de nós ($8,3 \pm 0,4$) e brotos ($12,6 \pm 0,8$) foram obtidos no meio MS com 1-NAA + TDZ ($0,1 + 1,0 \text{ mg L}^{-1}$), o maior comprimento de brotos ($6,0 \pm 0,5 \text{ cm}$) foi obtido no Meio MS com 2,4D + TDZ ($0,1 + 1,0 \text{ mg L}^{-1}$). O meio WPM com IBA + BAP ($1,0 + 0,1 \text{ mg L}^{-1}$) resultou em melhor enraizamento ($14,0 \text{ cm} \pm 1,3$) e número de raízes ($5,0 \pm 0,0$). O substrato vermiculita com areia promoveu 81,5% de plantas aclimatizadas. O estudo permitiu obter altos níveis de germinação, regeneração *in vitro* e aclimação de *S. alata* de alta qualidade genética.

Introduction

Plants are a source of biologically active compounds representing a genetic reservoir of great potential of new sources of natural, ornamental, cosmetic, and pharmaceutical products for human well-being (Monteiro and Brandelli 2017). In this context, the genus *Senna* (Fabaceae) stands out as it is well known in traditional medicine to treat diabetes, microbial infections, malaria, and other diseases. Recently a critical exploration of advances in the ethnopharmacology, phytochemistry, and toxicology of the plant species in the *Senna* genus has been done (Oladeji et al. 2021).

Senna alata (L.) Roxb. (sin. *Cassia alata*), popularly known as "candlestick" is a vital shrub widely and infections used in traditional medicine to treat diseases like ringworm, scabies, hemorrhoids, constipation, inguinal hernia, and intestinal parasites (Tangjitman et al. 2015; Andrade et al. 2018). Other studies report several more biological activities of *S. alata*, such as laxative, diuretic, phytoestrogenic, immunity stimulant, antifungal antibacterial and antioxidant activity (Promgool et al. 2014; De macedo et al. 2016). The economic interest of *S. alata* is growing every day due to the ample pharmacological activity (Fatmawati et al., 2020), Uses as an antibacterial antifungal and anticancer agent have been reported (Iraqui et al. 2019; Modarresi et al. 2021).

Here, plant tissue culture has improved the growth conditions of plants, from the use of plant growth regulators, becoming a powerful tool for cloning plants with healthy, vigorous and genetically superior characteristics that can multiply massively (Suman, 2017). Plant growth regulators (PGRs) are the synthetic analogs of plant hormones, many plant developmental processes can be actively regulated such as acceleration or delay of seed germination, stimulation or reduction of shoot elongation, dormancy breaking in woody perennials, induction of flowering and fruiting, reduction or increase of fruit set, acceleration or delay of senescence processes as well as fruit ripening and defoliation. (Dias, 2019).

The use of phytohormones in horticulture is a trend with great advantages growth regulators would be favorable as a good technique for the production of horticultural plants (Alcántara-Cortes et al. 2019). Environmental factors often exert inductive or inhibitory effects by evoking changes by hormones in metabolism and distribution within the plant. (Kumar et al. 2020, Bhattacharya, 2021).

To date, there are some reports on regeneration *in vitro* of *S. alata* (Ahmed et al. 2013; Thirupathi and Reddy, 2014), keeping in mind the use of *in vitro* approaches for the conservation of valuable medicinal plants. This study's objectives were to establish seedling germination methods and *in vitro* regeneration of *S. alata* using different plant growth regulators.

Material and Methods

The study was conducted at the Laboratory of Tropical Silviculture and Technologies Digital located at the National Institute for Research in the Amazon (LASTED / INPA) and the forest seedlings greenhouse of the University of the State of Amazonas (UEA-EST), Manaus, Brazil.

The seeds were obtained from seed bank, Laboratory of Microbiology and Soil Fertility. They were collected and registered at the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA) Registration number: 2008522 and in the Biodiversity Authorization and Information System (SISBIO) under number: 16702.

In vitro seed germination

Two experiments were carried out to evaluate aseptis and seed germination, following the methodology of Da Silva et al. (2018). In a first trial, we evaluated the most efficient concentration of sodium hypochlorite (NaOCl) for seed disinfection. The second experiment evaluated the effect of mechanical scarification, breaking dormancy to standardize and optimize germination.

Initially, seeds were rinsed for 1 minute with neutral soap; and were also immersed in 2.0 % (v/v) Carbendazim® fungicidal solution for 60 minutes. Subsequently, they were immersed in alcohol (70%) for 1 minute and, finally, in sodium hypochlorite (NaOCl) solutions of various concentrations (0.0, 0.10, 0.25, 0.50, and 1.0%) (v/v) for 30 minutes under agitation by a magnetic stirrer. The seeds were then washed four times with distilled water.

In the second experiment, the seeds were separated into two groups: one group was subjected to a pre-treatment of mechanical scarification with the aid of a marble stone until the visible wear of the integument on the side opposite the micropyle, the other group was used as control, under intact conditions. The seeds were inoculated using the methodology of Da Silva et al. (2018), which consisted of Murashige and Skoog (MS) culture medium, supplemented with sucrose (3 %) and agar-agar (0.7%). Thirty replicates were taken for each treatment, after 30 days seeds were evaluated for percentage of disinfection and germination seeds.

Shoot induction and multiplication

The MS and WPM media were supplemented with different cytokinins (6-benzylaminopurine (BAP) and Thidiazuron (TDZ) at a concentration of 1.0 mg L⁻¹ in combination with the auxins 1-naphthaleneacetic acid (1-NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) (0.1 mg L⁻¹). When starting the cultivation, we initially observed a strong oxidation at the ends of the explants where they were cut, consequently all explants died a few days after inoculation, including explants established in the control treatment without growth regulators.

To counteract the oxidation problem, subsequent explants (cotyledonary segments) were established in culture media supplemented with activated charcoal (2 mg L⁻¹). After 30 days of growth, the explants were evaluated in relation to the percentage of explants with shoot proliferation, number of shoots per bud, number of buds per stem, shoot height and callus and root formation.

In vitro rooting in microshoots

Regenerated shoots of about 4 to 5 cm in length were excised and transferred to the rooting media comprised of MS basal WPM medium supplemented with growth regulators, auxins (2,4-D, 1-NAA, indole-3-butyric acid (IBA) at a concentration of 1 mg L⁻¹, in combination with cytokinins (BAP and TDZ) at 0.1 mg L⁻¹. Shoot length, root length, and the number of roots data were recorded transferring onto the rooting medium after 60 days.

Culture media and conditions

Murashige and Skoog (MS) ® medium and Wood Plant Medium (WPM) ® supplemented with sucrose (3%) (w/v) and agar-agar (0.7%) (w/v), were used throughout the experiments. The medium's pH was adjusted to 5.8 using 1N NaOH or 1N HCl before autoclaving at 121°C at 1.06 kg cm⁻² pressure for 20 min. The explants were kept in a growth room with temperature of 25 ± 2 °C and 16 h of photoperiod with light intensity of 52 µmol·m⁻²·s⁻¹, under two cool white fluorescent lamps (GE.85W).

Hardening and acclimatization

Sprouts with a well developed root and shoot system were immersed in 2% Capriotop® fungicide

for 10 minutes then washed with sterile water. They were afterward transferred to sterile substrates, T1: vermiculite and vegetal soil (1:1) and T2: vermiculite and sand (1:1). The sprouts were maintained in greenhouse conditions for 90 days and then evaluated for the number of leaves, the number of roots, and percentage of survival.

Data collection and statistical analysis

The experiments of shoot induction, multiplication and rooting were conducted with a minimum of six replicate groups per treatment, and each replicate consisted of 5 experimental units (n = 30). Data were analyzed using Minitab® Statistical Software version 18. The data were subjected to analysis of variance (ANOVA), and the means were compared using the Tukey test with a 5% significance level, indicating that, for a significance level of 5%, MS medium was statistically better than WPM medium, confirmed by Tukey test .

Results and discussion

Regarding the asepsis test using different concentrations of sodium hypochlorite NaOCl (0.1 to 1.0%) a high percentage of seed disinfection was obtained, the treatment with NaOCl 0.5% allowed to obtain a percentage of 98.0% of disinfected seeds. and 2.0% contamination, this being the best result in terms of the concentrations used. (Figura 1). As for the scarification process, this was efficient for the germination process. The scarified seeds had a germination rate of 95.4%, while the intact seeds only had a 20% germination rate. During the first 4 days, scarified seeds reached 80% germination while intact seeds only 20% (Figure 2).

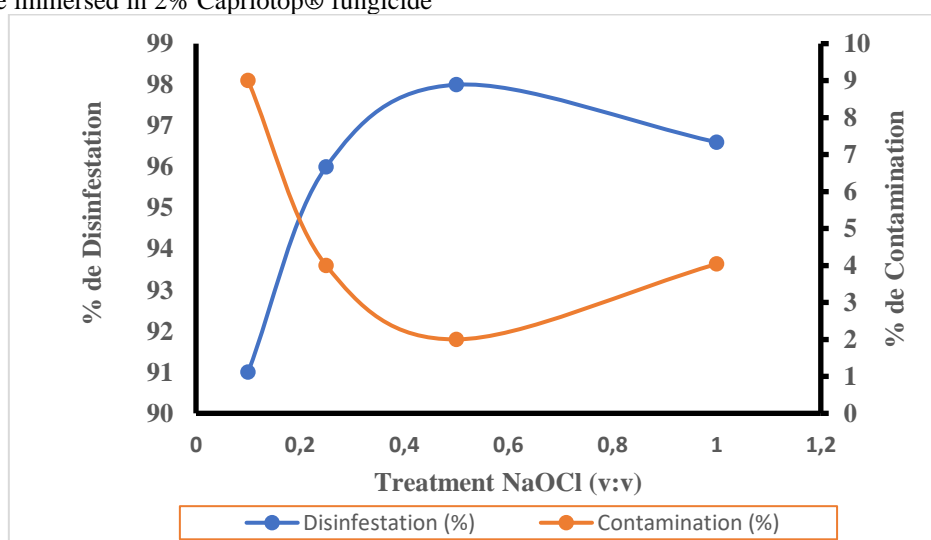


Figure 1. Disinfection of *S. alata* seeds with different concentrations of sodium hypochlorite (NaOCl).

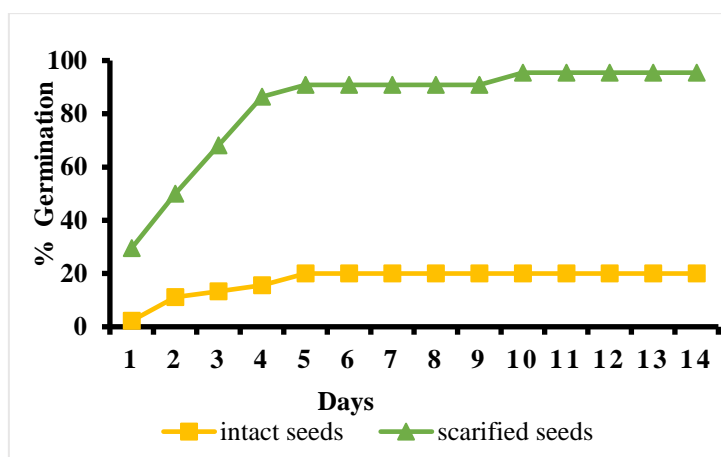


Figure 2. Germination of intact and scarified *S. alata* seeds in MS culture medium.

The effect of media was significant ($P \leq 0.05$) on shoot length of regenerated shoots (Table 1). The highest number of regenerated shoots (12.6 ± 0.8) and nodes (8.3 ± 1.0) was recorded on MS medium supplemented with 1-NAA and TDZ (0.1 and 1.0 mg L⁻¹, respectively). The lowest mean value for number of shoots (3.2 ± 0.7) and gems (3.1 ± 0.3) regenerated was obtained in WPM medium supplemented with 2,4D + TDZ (0.1 and 1.0 mgL⁻¹, respectively). The highest mean value for shoot length of (6.0 ± 0.5) (8.2 ± 0.5) was recorded in MS

medium supplemented with 2,4 D + TDZ (0.1 + 1.0 mg L⁻¹), after 30 days and 60 days followed by mean values of (7.8 ± 0.7) and (7.4 ± 0.6) recorded in MS media supplemented with 1-NAA + TDZ (0.1 + 1.0 mg L⁻¹) and 2,4D + BAP (0.1 + 1.0 mg L⁻¹ respectively). The lowest mean value for shoot length (0.8 ± 0.2) was obtained in pure WPM medium (control). There were significant differences in the mean shoot length values among the treatments (Table 1).

Table 1. Regeneration of *S. alata* from cotyledon nodal explants in MS and WPM media supplemented with BAP and TDZ in interaction with 2,4-D and 1-NAA.

Medium	Treatment (0,1 + 1,0 mg L ⁻¹)	Number of nodes/ 30 days	Number of shoots/30 days	Shoot length (cm) 30 days	Shoot length (cm) 60 days
MS	CONTROL	0.0 ± 0.0 ^d	0.0 ± 0.0 ^d	0.5 ± 0.2 ^d	2.8 ± 0.2 ^d
	1-NAA + TDZ	8.3 ± 0.4 ^a	12.6 ± 0.8 ^a	5.7 ± 0.6 ^a	7.8 ± 0.7 ^b
	2,4 D + TDZ	7.7 ± 0.3 ^b	10.7 ± 1.7 ^b	6.0 ± 0.5 ^a	8.2 ± 0.5 ^a
	2,4D + BAP	6.7 ± 0.4 ^b	9.7 ± 3.3 ^b	5.2 ± 0.4 ^b	7.4 ± 0.6 ^b
	1-NAA + BAP	5.6 ± 0.4 ^b	11.1 ± 0.6 ^b	5.1 ± 0.7 ^b	7.1 ± 0.7 ^b
WPM	CONTROL	0.0 ± 0.0 ^d	0.0 ± 0.0 ^d	0.8 ± 0.2 ^d	0.8 ± 0.2 ^d
	1-NAA + TDZ	3.1 ± 0.4 ^c	4.7 ± 0.6 ^c	4.4 ± 0.3 ^c	6.5 ± 0.3 ^c
	2,4D + TDZ	3.1 ± 0.3 ^c	3.2 ± 0.7 ^c	4.4 ± 0.2 ^c	6.8 ± 0.6 ^c
	2,4D + BAP	3.4 ± 0.5 ^c	3.5 ± 0.6 ^c	4.4 ± 0.7 ^c	6.5 ± 0.5 ^c
	1-NAA + BAP	3.2 ± 0.4 ^c	3.3 ± 0.9 ^c	4.8 ± 0.4 ^c	6.8 ± 0.4 ^c

The same letter averages do not differ at the level of 5% by the Tukey test.

As mentioned by Ahmed (2014), The presence of BAP, TDZ and 2,4D, ANA in WPM and MS media have synergistic effects on shoot multiplication when combining cytokinins compared to pure WPM and MS. Mean while, in this study, the medium supplemented with MS performed better than WPM for the variables evaluated induction and multiplication of shoots.

Our results showed that the optimum of cytokinin-auxin combinations on supplemented medium; effectively triggered shoot multiplication and elongation in *S. alata*. Similar investigations, using different plant growth regulators (PGR) have

been reported on *Cassia sophera* (Parveen and Shahzad 2014), *Cassia angustifolia* (Parveen and Shahzad 2011; Siddique et al. 2013), *Cassia siamea* (Parveen et al. 2010).

Although the number of shoots obtained in other *in vitro* regeneration studies of *S. alata* are significant. Rafique et al. (2013) reported a number of shoots (9.6 ± 0.3) and length of shoots (1.7 ± 0.1 cm) in the combination (7.5 μM) BA and (0.5 μM) indole-3-butyric acid (IBA). On the other hand Thirupathi and Reddy (2014) reported a number of shoots (4.1) and shoot length (3.51 ± 0.18 cm) in the combination of benzyladenine (BA) 6.6 μM and 1-

NAA (5.3 μM) Ahmed et al. (2013) resulted in a higher number of shoots per explant (18.9 ± 1.1) and shoot length (4.7 ± 0.1 cm) when the explants were grown in the presence of TDZ + BA (both 1.0 μM). By the methodology used in this study, I guarantee

to optimize the regeneration protocol obtaining a number of shoots (12.6 ± 0.8) and length of shoots (6.0 ± 0.5 cm) using the MS medium containing 1-NAA + TDZ (0.1 + 1.0 mg L^{-1}).



Figure 3: The *in vitro* regeneration process of *S. alata*. A) initial of the formation of shoots, MS medium supplemented with 2,4D + TDZ (0.1 + 1.0 mg L^{-1}) B) Subculture of shoots in the MS medium supplemented with 2,4D + TDZ (0.1 + 1.0 mg L^{-1}). C) Rooting of seedlings in MS medium supplemented BAP + IBA (1.0 \pm 0.1 mg L^{-1}). Normal plant after 120 days of culture.

Explants grown in medium WPM medium supplemented with IBA + BAP (1.0 + 0.1 mg L^{-1}) produced the most considerable number of roots per explant (5.0 ± 0) and root length (14.0 ± 1.3 cm) observed in the treatments. The IBA ensured better rooting in MS and WPM medium with the formation of long roots and a more significant number of

secondary roots in 4 weeks of cultivation (Figure 3 C) (Table 2). The auxin IBA has been widely used in promoting the rooting of other species of the genus *Cassia*, *Cassia alata* (Thirupathi and Reddy 2014) and *Cassia sophora* (Parveen and Shahzad 2014), *Cassia siamea* (Parven et al. 2010).

Table 2. Rooting of *S. alata* after 30 days of inoculation in MS and WPM medium supplemented with BAP and TDZ in interaction with 2,4-D, 1-NAA, and IBA.

Medium	Growth Regulators (1,0 + 0,1mg L ⁻¹)	Root length (cm)	Number of roots	Number of leaves
MS	2,4 D + TDZ	11.0 ± 0.8^b	4.4 ± 0.4^a	11.6 ± 0.5^a
	1-NAA + TDZ	12.6 ± 1.4^b	4.5 ± 0.5^b	11.3 ± 0.9^a
	2,4 D + BAP	9.3 ± 1.2^c	4.3 ± 0.4^b	11.3 ± 0.8^a
	1-NAA + BAP	13.8 ± 1.4^a	4.5 ± 0.5^b	11.0 ± 0.8^a
	IBA + TDZ	13.0 ± 2.0^a	4.4 ± 0.4^b	11.6 ± 0.8^a
	IBA + BAP	14.0 ± 1.2^a	4.7 ± 0.6^a	11.6 ± 0.9^a
WPM	2,4 D + TDZ	9.6 ± 1.3^c	4.8 ± 0.8^a	9.2 ± 0.5^b
	1-NAA + TDZ	14.0 ± 1.3^a	4.2 ± 0.8^c	9.5 ± 0.8^b
	2,4 D + BAP	12.5 ± 1.5^b	3.8 ± 0.6^d	9.0 ± 0.61^b
	1-NAA + BAP	13.7 ± 1.3^a	4.2 ± 0.7^b	7.9 ± 0.61^c
	IBA + TDZ	10.3 ± 1.3^c	4.7 ± 0.5^a	6.9 ± 0.7^c
	IBA + BAP	14.0 ± 1.3^a	5.0 ± 0.0^a	10.6 ± 0.9^b

The same letter averages do not differ at the level of 5% by the Tukey test

The type of explant has an essential effect on the *in vitro* regeneration rates, as different tissues may have different levels of endogenous hormones. This fact was observed in this study. The cotyledon segments demonstrated to be efficient in regeneration. In general, nodal explants and

cotyledon explants proved to be excellent tissues for achieving a high frequency of regeneration and multiplication of shoots (Ahmed 2014).

Acclimatization.

On acclimatization, there were no significant differences observed in the treatments analyzed. Seedlings with well-developed leaves and roots were successfully acclimatized under nursery conditions. Among the substrates used, the vermiculite and sand substrate promoted the highest acclimated seedling survival rate of 81.5% (Table 4).

It was further noted that growth variables such as the number of leaves, the length, and the number of developed roots positively influenced the plant's survivorship (Figure 4). This outcome may be related to the development of the leaf structure in the acclimatization phase, which resulted in healthy and

uniform seedlings and subsequent improved food production in the plant healthy and well-developed plants after 1 year in the housegarden (Figure 5).

Several similar survival results have been reported in the acclimatization of *S. alata* when testing different types of substrate. Thiruparti et al. (2014), while using land-manure from a farm and sand (1:1:1), obtained 81% acclimatized plants. Ahmed et al. (2013) reported an 85% survival when using Soilrite® peat-vermiculite moss (1:1:1), while Parveen et al. (2010) reported 85% survival using garden manure soil.

Table 4. Average values of survival (%) obtained in the treatments of acclimatization of seedlings of *S. alata*.

Treatment	Survival	Grouping
T2 (Vermiculite + sand)	81.5 %	A
T1 (Vermiculite + soil)	75.08 %	A

Averages followed by the same letter do not differ at the level of 5% by the Tukey test

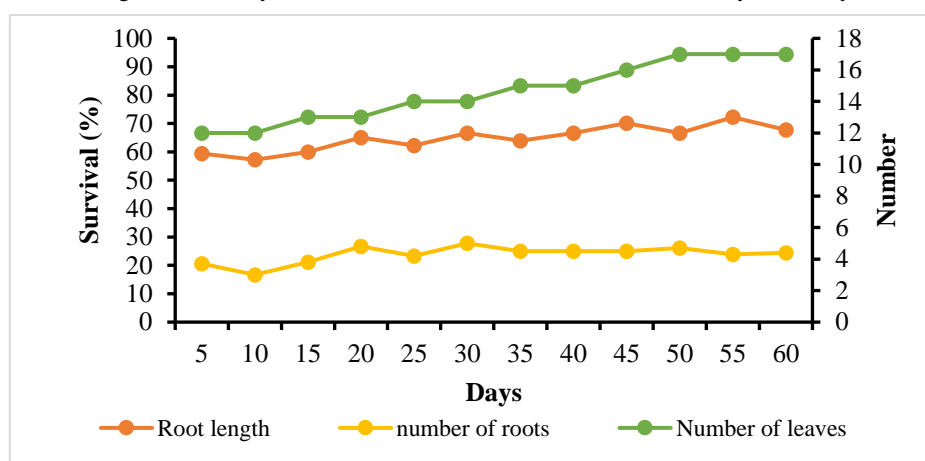


Figure 4. Survival and growth of *S. alata* seedlings in the process of acclimatization



Figure 5. Developed plant of *S. alata* originated from *in vitro* cultivation after 1 year of acclimatization greenhouse.

Conclusions

The results of this study illustrate germination from seeds, in addition regeneration from nodal segments that allow rapid multiplication of shoots and *in vitro* rooting of *S. alata*. MS culture medium supplemented with growth regulators such as cytokinins (BAP, TDZ) in combination with auxins (2,4-D, AIB and ANA), in addition to activated charcoal, allow organogenesis in the different *in vitro* regeneration phases. In conclusion, an efficient and effective procedure was developed for the *in vitro* germination and regeneration of *S. alata*. This procedure provides a successful and rapid technique that allow and promote organogenesis at different stages of regeneration of *S. alata*, a leguminous of great medicinal importance.

Abbreviations

BAP, 6 Benzylaminopurine; TDZ, N-phenyl-1,2,3-thiazol-5-ylurea; 2,4-D, 2,4-dichlorophenoxyacetic acid; IBA, indole-3-butyric acid; 1-NAA, 1-naphthalene acetic acid;; MS,

Murashige and Skoog medium; WPM, Wood Plant medium PGR, plant growth regulator

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